Depletion of SecDF-YajC causes a decrease in the level of SecG: implication for their functional interaction

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Abstract SecA and an apparatus comprising SecYEG and SecDF-YajC complexes catalyze protein translocation across the *Escherichia coli* membrane. SecDF-YajC and SecG facilitate membrane insertion of SecA, which is the driving force for protein translocation. Here we report that SecDF-YajC depletion together with SecG depletion nearly completely inhibits protein translocation both in vivo and in vitro, although SecDF-YajC had been thought to be unnecessary for in vitro translocation. The level of SecG in membranes decreased to about half upon SecDF-YajC depletion and recovered to a normal level when SecDF-YajC was expressed. SecDF-YajC inhibited disulfide bond formation between two SecG molecules possessing a single cysteine residue. These results suggest functional interaction between SecDF-YajC and SecG.

Key words: Protein translocation; Sec machinery; SecDF-YajC; SecG; Inverted membrane vesicle

1. Introduction

Recent studies have revealed that *Escherichia coli* protein translocase is composed of a heterotrimeric SecYEG complex, to which another heterotrimeric complex, SecDF-YajC, binds [1–5]. The genes encoding YajC, SecD and SecF form an operon [6,7]. Disruption of the *yajC* gene has no effect on protein translocation [7]. SecA undergoes a conformational change upon binding and hydrolysis of ATP [1–5], and delivers a precursor protein into a protein-conducting channel, which is proposed to be formed through oligomerization of the SecYEG complex [8]. Both SecG [9,10] and SecDF-YajC [10] facilitate the SecA function. It has been proposed that the conformational change of SecA is facilitated by membrane topology inversion of SecG [11]. SecDF has been proposed to be important for the release of translocated proteins from the translocase [12], and for the prevention of backward sliding of the translocated proteins [13]. Cells lacking SecD and SecF are severely defective and hardly viable [14]. Despite these facts, depletion of SecDF has no effect on protein translocation into inverted membrane vesicles (IMVs) [15] or reconstituted proteoliposomes [16]. Depletion of SecG causes a defect in protein translocation at low temperatures [17] or in the absence of the proton motive force [18], whereas protein translocation at 37°C is only slightly defective in the absence of SecG [19].

To further understand the auxiliary functions of SecDF-YajC and SecG, these proteins were depleted together or separately. We show here that the simultaneous depletion of these proteins results in very severe inhibition of protein translocation not only in vivo but also in vitro. Thus, protein translocation into IMVs becomes strongly dependent on SecG when SecDF-YajC is depleted. Moreover, the results suggest that SecG functionally interacts with SecDF-YajC.

2. Materials and methods

2.1. Bacteria and plasmids

E. coli BL325 (BL21 *tgt::kan-araC*$^{+}$-P_{BAD}::*yajCsecDF*) [10] was a generous gift from Franck Duong. To construct BL325-YK (BL325 Δ*secG::cat*), the Δ*secG::kan* allele carried by pDG41 [17] was replaced by the *cat* gene encoding the chloramphenicol resistance gene. The plasmid, pDG41cat, thus constructed was linearized with *Bam*HI and *Sph*I, and then transformed into FS1576 [20]. Transformants that were sensitive to tetracycline and resistant to chloramphenicol were selected. The Δ*secG::cat* gene of one transformant was introduced into BL325 by means of P1 transduction to construct BL325-YK. Plasmids encoding wild-type SecG or three SecG-Cys derivatives, 3.5C, 82C and 88C, under the control of the *tac* promoter were constructed by cloning the *Bam*HI-*Sal*I fragment of plasmids encoding the respective SecG-Cys derivatives [21] at the *Bam*HI-*Xho*I sites of pAT1. To construct pAT1, a *Pst*I-*Bam*HI fragment of pUSI2 [22] carrying *lacI* and the *tac* promoter was ligated with a *Pst*I-*Bam*HI fragment of pKQ2 [17] containing the replication origin of pSC101 [23] and the *bla* gene.

2.2. Materials

Tran^{35}S-label (37 TBq/mmol as [^{35}S]Met) was obtained from ICN. Antibodies were raised in rabbits against OmpA [24], SecY [25], SecE [12], SecG [26], SecD [12], and SecF [26] as reported. SecA [27] and the non-radioactive precursor of outer membrane protein A (proOmpA) [28] were purified as reported.

2.3. In vivo and in vitro proOmpA translocation

In vivo processing of proOmpA to OmpA was examined at 37°C by means of pulse-chase experiments as described [12]. Cells were pulse-labeled with Tran^{35}S-label (20 μCi/ml) for 30 s and then chased by the addition of 12 mM each of cold methionine and cysteine.

IMVs were prepared as described [29]. [^{35}S]ProOmpA was synthesized in vitro as described [24]. The reaction mixture (25 μl), comprising 0.2 mg/ml IMVs, 1 mM ATP, 1 mM $MgSO_4$, [^{35}S]proOmpA, 25 μg/ml cold proOmpA, 50 μg/ml SecB, 60 μg/ml SecA, an ATP-generating system comprising 2.5 mM creatine phosphate and 5 μg/ml creatine kinase, and 50 mM potassium phosphate (pH 7.5), was incubated at 37°C for the indicated times. The reaction mixture was

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; proOmpA, precursor of outer membrane protein A (OmpA); OD, optical density; NEM, *N*-ethylmaleimide; IPTG, isopropyl β-D-thiogalactopyranoside; IMV, inverted membrane vesicle

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placed on ice and then treated with 1 mg/ml proteinase K for 30 min to terminate the reaction. OmpA was precipitated with 10% trichloroacetic acid, successively washed with acetone, and then analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and fluorography. The translocation activity was determined by densitometric quantification of proteinase K-resistant OmpA with an ATTO Densitograph.

2.4. SDS–PAGE and Western blot analyses

SecG was analyzed by SDS–PAGE on a gel composed of 12.5% acrylamide and 0.27% *N*,*N*′-methylenebisacrylamide as described [30]. Other proteins were analyzed by SDS–PAGE according to Laemmli [31]. Immunoblotting was performed as described [32].

3. Results

3.1. Phenotype of the yajCsecDF-secG null mutant

The *E. coli* BL325 strain carries the chromosomal *yajC-secD-secF* operon under the control of P_{BAD} [10]. The *secG* null mutation (Δ*secG::cat*) was introduced into BL325 to construct BL325-YK (P_{BAD} *yajC-secDF* Δ*secG::cat*). The BL325 and BL325-YK strains were grown overnight at 37°C on LB plates in the presence or absence of 0.2% arabinose (Fig. 1A). As reported [10,14], BL325 exhibited a severe growth defect in the absence of arabinose (depletion of SecDF-YajC) even at 37°C, whereas SecG depletion alone had little inhibitory effect on growth. In contrast, BL325-YK did not grow at all in the absence of arabinose, indicating that the depletion of SecDF-YajC together with SecG depletion strongly inhibited the growth of *E. coli*. However, both strains slowly grew on liquid media such as M63 or LB containing no arabinose, and the culture optical density (OD) reached ~1.0. We could therefore examine the processing of proOmpA into mature OmpA by means of pulse-chase experiments at 37°C in BL325 and BL325-YK cells grown on M63 (Fig. 1B). SecDF-YajC depletion caused only slight inhibition of proOmpA processing and SecG depletion had almost no effect at all. In marked contrast, the depletion of both SecDF-YajC and SecG resulted in a very severe defect in proOmpA processing, suggesting the synergistic effect of SecDF-YajC and SecG depletion.

3.2. Effect of SecDF-YajC-SecG depletion on in vitro protein translocation

The BL325 and BL325-YK strains were grown on LB at 37°C in the presence and absence of 0.2% arabinose. IMVs were prepared from the cells harvested at OD 0.8. When IMVs prepared from cells grown in the absence of arabinose were examined, SecD and SecF were hardly detected on immunoblotting (see Fig. 4). Although SecA expression was derepressed by SecG or SecDF-YajC depletion [6,17], the level of SecA was essentially the same in all IMVs (data not shown). SecDF-YajC depletion has been reported to cause inhibition of proton motive force generation [15], although a paper refuting this has appeared [33]. We found that IMVs lacking SecDF-YajC were unable to generate the proton motive force through F_0F_1-ATPase (data not shown). In order to compare the translocation activities of IMVs in the absence of the proton motive force, proOmpA translocation was examined in the presence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Fig. 2A,B). The initial rates of proOmpA translocation were determined under the respective conditions (Fig. 2C,D). SecDF-YajC depletion alone had no effect on proOmpA translocation (compare the open and closed circles)

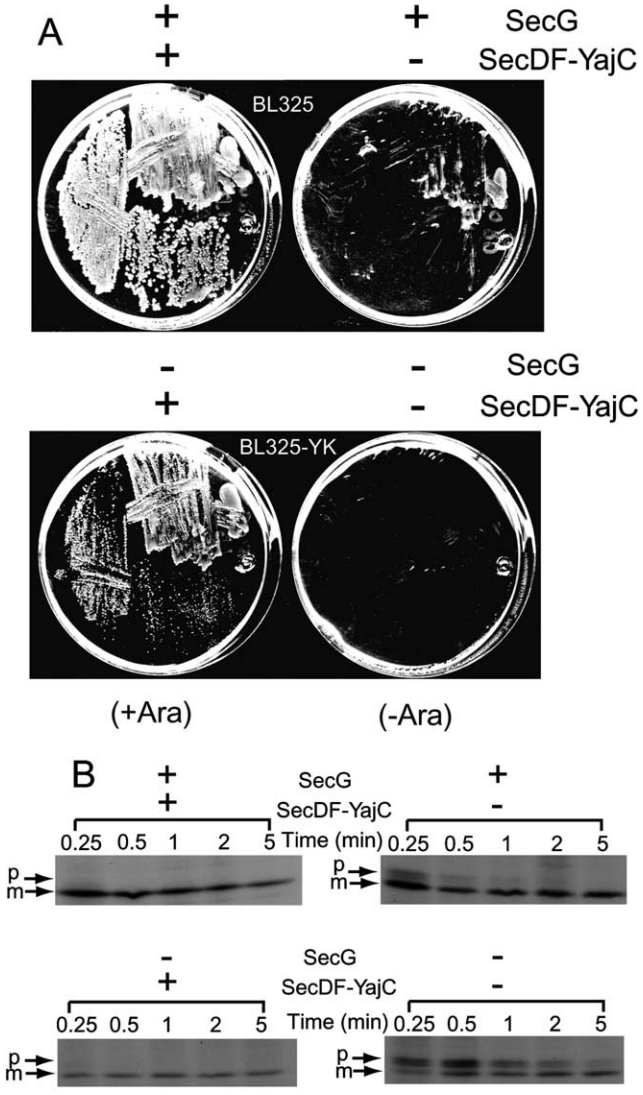

Fig. 1. Simultaneous depletion of SecDF-YajC and SecG causes severe inhibition of protein translocation. A: The *E. coli* BL325 (P_{BAD}::*yajCsecDF*) and BL325-YK (BL325 Δ*secG::cat*) strains were grown at 37°C overnight on LB supplemented with 0.2% arabinose. After washing three times with fresh LB, BL325 (upper panel) and BL325-YK (lower panel) cells were inoculated onto LB plates containing (+Ara) or not containing (−Ara) 0.2% arabinose and then grown overnight at 37°C. B: BL325 and BL325-YK cells were grown overnight on M63 medium containing 0.2% arabinose. The harvested cells were washed three times with fresh M63 and then grown at 37°C on M63 with or without 0.2% arabinose until the OD reached 0.8. The cells were pulse-labeled with Tran^{35}S-label, and then chased with non-radioactive methionine and cysteine for the indicated times. Mature OmpA (m) and proOmpA (p) were immunoprecipitated with anti-OmpA antibodies, and then analyzed by SDS–PAGE and fluorography.

whether SecA was exogenously added or not. This is consistent with the observation that SecDF-YajC is not required for in vitro protein translocation [15,16]. On the other hand, as reported previously [18], SecG depletion inhibited proOmpA translocation even at 37°C, because the proton motive force was not generated (compare the open circles and triangles). Strikingly, however, when SecDF-YajC and SecG were depleted together (closed triangles), the translocation was near completely inhibited even in the presence of external SecA.

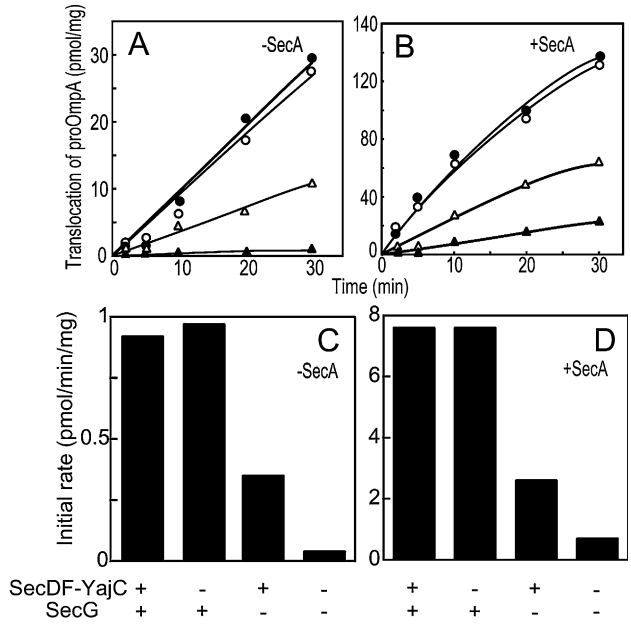

Fig. 2. Effects of SecDF-YajC depletion on in vitro proOmpA translocation in the presence or absence of SecG. IMVs were prepared from the BL325 (circles) and BL325-YK (triangles) strains, which were grown on LB in the presence (open symbols) and absence (closed symbols) of arabinose as described in Fig. 1B except that LB was used instead of M63. Translocation of proOmpA into IMVs was examined at 37°C in the presence of 20 μM CCCP with (B) or without (A) 60 μg/ml external SecA as described in Section 2. The initial rates of proOmpA translocation under the respective conditions were determined (C,D).

These results indicate that the translocation activity in the absence of SecDF-YajC is strongly dependent on SecG (compare the closed circles and triangles). It was also revealed that SecDF-YajC is required for in vitro translocation if SecG is absent. Taken together, these results suggest that SecDF-YajC and SecG have complementary functions.

3.3. Effect of SecDF-YajC depletion on the level of SecG

Interaction between Sec factors often causes stabilization of the interacting partner, for example, stable expression of SecY depends on the simultaneous expression of SecE [34], and an unstable SecE derivative requires SecY or SecG for its stabilization [32,35]. Since SecDF-YajC and SecG appeared to interact functionally, we examined whether or not SecDF-YajC depletion affects the level of SecG. The BL325 strain grown overnight in the presence of arabinose was inoculated into fresh medium containing no arabinose. The cells were harvested at various times after the start of SecDF-YajC depletion, and then subjected to SDS–PAGE and immunoblotting (Fig. 3). SecD became undetectable at 2.5 h, whereas the level of SecY remained almost unchanged, even at 6.5 h. The level of SecE was also constant except for the last point. Strikingly, however, the level of SecG continuously decreased to about 50% of that found at time 0 (Fig. 3B). When the strain was cultivated in the presence of arabinose, the levels of all Sec factors remained normal (see open symbols at 5 h). The level of SecD was essentially the same between BL325 grown in the presence of arabinose and its parental strain BL21 (data not shown). These results suggest that about 50% of the SecG molecules are stabilized by SecDF-YajC.

3.4. Levels of Sec proteins in IMVs

The levels of Sec factors in the IMVs used in Fig. 2 were examined by SDS–PAGE and quantitative immunoblotting (Fig. 4). The level of SecE was appreciably decreased by the depletion of both SecG and SecDF-YajC. This might have caused a slight decrease in the level of SecY in the absence of SecG and SecDF-YajC. The amount of SecG in IMVs was decreased to about 50% by the depletion of SecDF-YajC. In contrast, SecG depletion alone did not cause decreases in the amounts of other Sec factors, as reported [36]. Therefore, the decrease in protein translocation activity observed upon SecG depletion (Fig. 2) is solely caused by the absence of SecG.

3.5. SecDF-YajC expression decreases the disulfide-bonded SecG dimer

SecG, comprising 110 amino acid residues, spans the membrane twice, and its N- and C-termini are both exposed to the periplasm [11]. It has been reported that SecG-Cys derivatives possessing a single cysteine residue in the C-terminal region form a SecG dimer through disulfide bonds [21], suggesting that two SecG molecules exist close to each other in a single translocation machinery. Since SecDF-YajC depletion decreased the SecG level to about 50%, the effect of the deple-

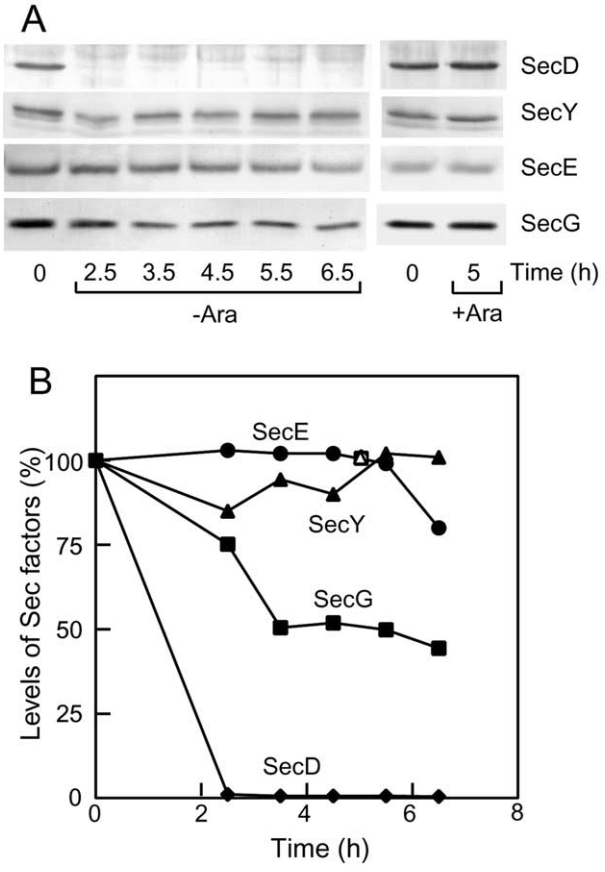

Fig. 3. Depletion of SecDF-YajC specifically decreases the level of SecG. The BL325 strain was grown overnight at 37°C on LB medium containing 0.2% arabinose. The cells were harvested, washed three times with fresh LB, and then grown at 37°C on LB with (open symbols) or without (closed symbols) arabinose. Portions of the cultures were withdrawn at the indicated times, and then the specified Sec proteins were analyzed by SDS–PAGE and immunoblotting (A). The blots shown in A were quantitated with an ATTO Densitograph. The amounts of the respective proteins are expressed as percentages, taking the amount at time 0 as 100% (B).

tion on the dimerization of SecG-Cys derivatives was examined (Fig. 5). SecG-Cys derivative 3.5C possesses a cysteine residue introduced between residues 3 and 4. The residues at positions 82 and 88 exposed to the periplasm are replaced by cysteine in SecG-Cys derivatives 82C and 88C, respectively. BL325-YK cells harboring a plasmid encoding wild-type SecG or one of the indicated SecG-Cys derivatives under the control of the *tac* promoter were grown at 37°C overnight in the presence of 0.2% arabinose to allow normal growth. The cells were harvested and then subjected to SecDF-YajC depletion for 3.5 h in fresh medium containing no arabinose. Isopropyl β-D-thiogalactopyranoside (IPTG, 1.5 mM) was added at time 0 to induce SecG-Cys derivatives for 1 h in the absence of SecDF-YajC. SecDF-YajC was then induced by the addition of 0.2% arabinose. At the indicated time points, portions of the cultures were withdrawn and immediately treated with *N*-ethylmaleimide (NEM) to block free SH. Cellular proteins were analyzed by SDS–PAGE in the presence and absence of β-mercaptoethanol, followed by immunoblotting with anti-SecG antibodies. Only monomeric SecG was detected with wild-type SecG even in the absence of β-mercaptoethanol. Essentially all 3.5C molecules existed as a monomer in the absence of SecDF-YajC expression. The amount of the 3.5C dimer increased only marginally upon the expression of SecDF-YajC. In contrast, significant portions of both 82C and 88C expressed for 1 h in the absence of SecDF-YajC existed as dimers that were converted into monomers on treatment with β-mercaptoethanol. Strikingly, the amounts of the 82C and 88C dimers decreased with the period of SecDF-YajC induction. Furthermore, the total amounts of 82C and 88C determined after the reduction of disulfide bonds remained constant whereas those of wild-type SecG and 3.5C increased with the induction of SecDF-YajC. The levels of SecG and SecG-Cys derivatives expressed at 4 h were essentially the same as the normal level of wild-type SecG expressed from the chromosome (see the left lanes of the respective blots). These results suggest that the disulfide-linked SecG-Cys dimer is stable even in the absence of SecDF-YajC, while the arrangement of SecG-Cys molecules in the translocase is affected by the expression of SecDF-YajC.

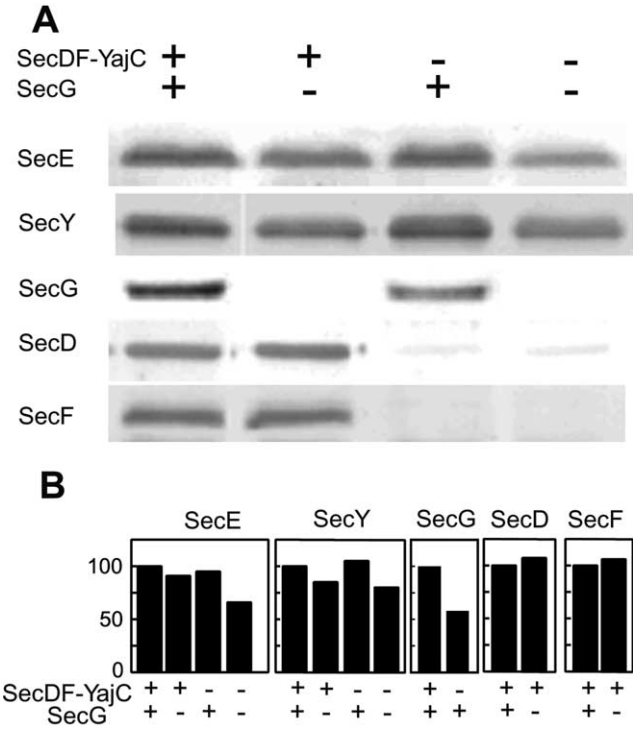

Fig. 4. The amounts of the indicated Sec factors in IMVs were determined by SDS–PAGE and immunoblotting. A: For analysis of SecY, SecE and SecF, 20 μg of the IMVs used in Fig. 2 were examined. For SecG and SecD, 0.3 and 2 μg of IMVs were analyzed, respectively. B: The amounts of the indicated Sec proteins were determined and expressed as percentages, taking the amounts of the respective proteins shown in the left lane of A as 100%.

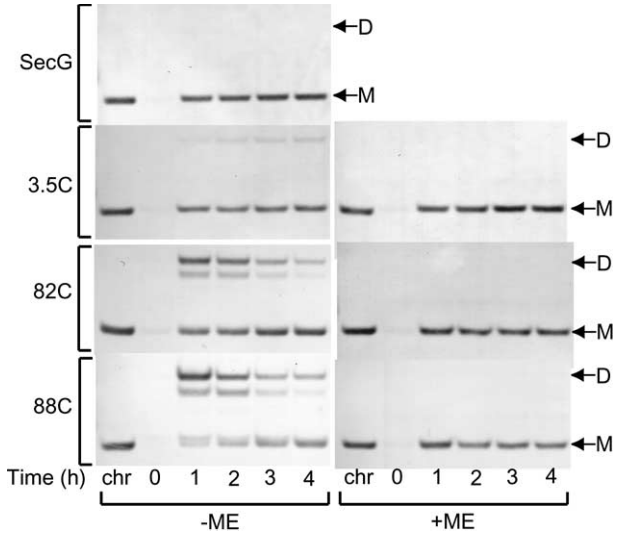

Fig. 5. Effect of SecDF-YajC expression on the formation of the SecG-Cys dimer. BL325-YK harboring a plasmid encoding wild-type SecG or one of the indicated SecG-Cys derivatives under the control of the *tac* promoter was grown overnight on LB supplemented with 0.2% arabinose. The cells were washed three times with fresh LB containing no arabinose. SecDF-YajC was depleted by cultivating cells on LB containing no arabinose at 37°C for 3.5 h, and then 1.5 mM IPTG was added to express SecG or one of its derivatives at 0 time. Arabinose (0.2%) was added at 1 h to induce SecDF-YajC. Portions of the cultures were withdrawn at the indicated times and treated with 10 mM NEM for 30 min. Cellular proteins were treated with SDS–PAGE sample buffer with or without β-mercaptoethanol (ME), and then analyzed by SDS–PAGE and immunoblotting with anti-SecG antibodies to detect monomeric (M) and dimeric (D) SecG. As a control, the level of SecG expressed from the chromosome of BL325 cells, which were grown overnight in the presence of arabinose, was examined in the left lane of each blot (chr). Equal amounts of cellular proteins were analyzed by SDS–PAGE.

4. Discussion

Depletion of both SecDF-YajC and SecG caused near complete inhibition of protein translocation even at 37°C, suggesting that the function of SecA is severely restricted by the complete loss of these auxiliary factors. Thus, SecG was almost completely indispensable for protein translocation in the absence of SecDF-YajC. It has been observed that the stimulation of protein translocation by SecG is significantly higher in proteoliposomes reconstituted with SecY and SecE [26,37] than in IMVs [18]. The absence of SecDF-YajC in these proteoliposomes most likely accounts for the strong SecG dependence. SecDF-YajC has been thought to be dispensable for in vitro protein translocation [15,16]. However, this complex was also found to be important for the translocation activity in the

absence of SecG, although the stimulation by SecDF-YajC was weaker than that by SecG. The stimulation of protein translocation by SecDF-YajC and SecG shown here is consistent with their stimulation of the membrane insertion of SecA [9,10].

E. coli protein translocase has been extensively studied genetically, biochemically and biophysically [1–5]. These studies revealed complex and dynamic properties of the translocase. It is now established that the heterotrimeric SecDF-YajC complex interacts with the heterotrimeric SecYEG complex [10]. It is also proposed that the SecYEG complex multimerizes upon protein translocation [8]. However, little is known about how the two complexes interact with each other, and how this interaction is related to SecYEG multimerization. The numbers of SecYEG and SecDF-YajC complexes in a single cell must differ significantly because of the difference in molecular numbers of the subunits constituting the respective complexes [7,16,35]. Thus, the stoichiometry of the two complexes also remains to be clarified. It seems unlikely that the decrease in the SecG level upon SecDF-YajC depletion is caused by defective membrane insertion of SecG, since the dimerization of SecG through disulfide bonds occurs on the periplasmic side of the membrane. The levels of Sec factors in the membrane are thought to be tightly regulated. Proteolysis of Sec factors, which are not in the translocase, plays an important role in this regulation [38]. As to the level of SecYEG, a decrease in the level of SecE due to the *secEcs501* mutation causes decreases in the levels of both SecY [26,39] and SecG [26]. It has been proposed that SecG is peripherally located in the three-dimensional structure of SecYEG [40]. Furthermore, it has been reported that the SecY and SecE subunits in the assembled SecYEG complex are not exchanged with the respective newly synthesized subunits whereas SecG in the complex is exchanged [41]. Taken together, these observations and the results presented here suggest that SecG is located at the interface between the SecYEG multimer and SecDF-YajC, thereby interacting with not only SecYE but also SecDF-YajC. This seems to be the reason why about half of the SecG molecules require SecDF-YajC expression for their stabilization, although further studies are necessary to show the direct interaction between SecG and SecDF-YajC. It should be noted that the SecDF-YajC complex may mediate interaction between the SecYEG complex and YidC, which is involved in membrane protein insertion [42].

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